FORM PTO-1390 (REV. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: REG 334-A-US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/009620 NOT YET KNOWN

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/15732	June 8, 2000	June 17, 1999

TITLE OF INVENTION: METHODS OF IMAGING AND TARGETING TUMOR VASCULATURE

APPLICANT(S) FOR DO/EO/US: STANLEY J. WIEGAND

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [X] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: Express Mail Label No. ET712522476US dated December 11, 2001

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Not Yet Known 10/009620	PCT/US00/15732	REG 334-A-US

	CALCULATIONS PTO USE ONLY	
21. [X] The following fees are submitted:		
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 740.	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	67 - 20 =	47	x **$18.00**	$ 846.	
Independent claims	6 - 3 =	3	x **$84.00**	$ 252.	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$ 280.	
TOTAL OF ABOVE CALCULATIONS =				$ 2,118.	
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$ 2,118.	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 2,118.	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 2,118.	
				Amount to be refunded:	$
				charged:	$

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 18-0650 in the amount of $ 2,118. to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-0650 . A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Att. Docket No. REG 334-A-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application Of	:	Stanley J. Wiegand
USSN	:	Not Yet Known
Filed	:	Filed Herewith
Int'l File No.	:	PCT/US00/15732
Int'l File Date	:	June 8, 2000
For	:	METHODS OF IMAGING AND TARGETING TUMOR VASCULATURE
Examiner	:	Not Yet Known
Group	:	Not Yet Known

December 11, 2001

Commissioner for Patents
U.S. Patent and Trademark Office
Washington, D.C. 20231

Att:

PRELIMINARY AMENDMENT

Sir:

This paper is submitted in connection with the above-identified application. Prior to examination of the application on the merits, please amend the specification as follows:

In the Specification:

Please replace the paragraph starting on page 1, line 3, with the following:

This application claims priority to International Application No. PCT /US00/15732, filed June 8, 2000, which claims priority to U.S. Provisional Application No. 60/139,642, filed June 17, 1999. Throughout this application various publications are

referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

REMARKS

This Preliminary Amendment is made merely to add priority date to the application. Applicant submits herewith as Exhibit A: Marked-Up Version of Page 1 (1 sheet); and Exhibit B : formal drawings (5 sheets).

No fee is deemed necessary for filing this paper. However, if any fees are deemed necessary, the Commissioner is hereby authorized to charge any such fees required by this paper to Deposit Account No. 18-0650.

Respectfully submitted,

Linda O. Palladino

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MARKED-UP VERSION
5 PRTS

METHODS OF IMAGING AND TARGETING TUMOR VASCULATURE

This application claims priority to International Application No. PCT/US00/15732, filed June 8, 2000, which claims priority to U.S. Provisional Application No. 60/139,642, filed June 17, 1999.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

INTRODUCTION

The field of this invention is methods of imaging and targeting tumor vasculature. Specifically, the field of this invention relates to using Angiopoietin-2 (Ang-2) to image developing tumor or tumor associated vasculature. It also relates to using Ang-2 to target chemotherapeutic agents to developing tumor or tumor associated vasculature.

BACKGROUND

The growth and metastasis of tumors depends, in part, on their ability to induce the growth of new blood vessels (J. Folkman, N Engl J Med 285, 1182 (1971); J. Folkman, J. Natl. Cancer Instit. 82, 4 (1990); D. Hanahan and J. Folkman, Cell 86, 353-364 (1996)). It is widely accepted that most tumors and metastases originate as small avascular structures, which must induce the development of new vessels in order to grow beyond a few millimeters in size (J. Folkman, N Engl J Med 285, 1182 (1971); J. Folkman, J. Natl. Cancer Instit. 82, 4 (1990)). An initial phase of avascular growth certainly seems to be a feature of tumor cells seeded into avascular structures such as the cornea, the anterior eye chamber or an artificial tumor window chamber, as well as of tumor cells implanted into virtual spaces, such those implanted

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subcutaneously or into the peritoneum (J. Folkman, J. Natl. Cancer Instit. 82, 4 (1990)). It also occurs when tumor cells grow on epithelia or tissue surfaces (J. Folkman, J. Natl. Cancer Instit. 82, 4 (1990); D. Hanahan and J. Folkman, Cell 86, 353-364 (1996)). However, other evidence suggests that, prior to the initiation of angiogenesis, many tumors may not grow in an avascular fashion, particularly within the confines of a vascularized tissue (P. Wesseling, J. A. van der Laak, H. de Leeuw, D. J. Ruiter, P. C. Burger, J Neurosurg 81, 902 (1994); L. Holmgren, M. S. O'Reilly, J. Folkman, Nature Medicine 1, 149 (1995); F. Pezzella, et al., Am J Pathol 151, 1417 (1997)). In such settings, tumor cells often seem to "home" to existing blood vessels. The interplay between such initial co-opting of existing vessels and subsequent tumor-induced angiogenesis has not been extensively examined.

Tumor-induced angiogenesis is thought to depend on the production of pro-angiogenic growth factors by the tumor cells, which overcome other forces that tend to keep existing vessels quiescent and stable (D. Hanahan and J. Folkman, Cell 86, 353-364 (1996)). Perhaps the best characterized of these pro-angiogenic agents is vascular endothelial growth factor (VEGF) (N. Ferrara and T. Davis-Smyth, Endocr Rev 18, 4 (1997)). Recently, a second family of growth factors specific for the vascular endothelium has been identified, with members of this family termed the Angiopoietins (S. Davis, et al., Cell 87, 1161 (1996); C. Suri, et al., Cell 87, 1171 (1996); P. C. Maisonpierre, et al., Science 277, 55 (1997); D. Valenzuela, et al., Proc Nat Acad Sci In press (1999)). As for VEGF, the specificity of the Angiopoietins for the vascular endothelium results from the restricted distribution of the Angiopoietin receptors, Tie1 and Tie2, to these cells. The Angiopoietins are the only known

growth factor family to contain both receptor activators, such as Angiopoietin-1 (Ang-1), as well as naturally occurring receptor antagonists, such as Ang-2 (see published PCT application WO 96/11269, published April 18, 1996 or published PCT application WO 96/31598, published October 10, 1996, both of which are incorporated by reference in their entireties), suggesting that turning off the Tie receptors might be just as critical as turning them on. VEGF and the Angiopoietins seem to play complementary and coordinated roles, which have been best characterized during development. Gene knockout studies in mice have proven that VEGF and one of its receptors, Flk1/KDR, are absolutely required for the early embryonic stages of vascular development involving endothelial cell differentiation, proliferation and primitive vessel formation (Figure 1A) (F. Shalaby, et al., Nature 376, 62 (1995); P. Carmeliet, et al., Nature 380, 435 (1996); N. Ferrara, et al., Nature 380, 439 (1996)). In contrast, in mouse embryos lacking either Ang-1 or Tie2, the early stages of VEGF-dependent vascular development seem to occur rather normally, resulting in the formation of a primitive vasculature (P. C. Maisonpierre, et al., Science 277, 55 (1997); D. J. Dumont, et al., Genes & Development 8, 1897 (1994); T. N. Sato, et al., Nature 376, 70 (1995)). However, remodeling and stabilization of this primitive vasculature is severely perturbed (Figure 1A), leading to embryonic lethality. These defects seem to result from aberrant interactions between endothelial cells and surrounding extracellular matrix and supporting elements such as smooth muscle cells (P. C. Maisonpierre, et al., Science 277, 55 (1997); J. Folkman, P. A. D'Amore, Cell 87, 1153 (1996)), leading to abnormal vascular remodelling as well as vessel regression. Transgenic overexpression of Ang-1 leads to striking

hypervascularization, presumably by promoting vascular remodeling events and perhaps by decreasing normal vascular pruning (C. Suri, et al., Science 282, 468 (1998)). Consistent with the notion that Ang-2 acts as a natural antagonist for the Ang-1/Tie2 interaction, transgenic overexpression of Ang-2 during embryogenesis (P. C. Maisonpierre, et al., Science 277, 55 (1997)) leads to a lethal phenotype reminiscent of that seen in embryos lacking either Ang-1 or Tie2, with severe disruptions in vascular development (Figure 1A).

In endothelial cells Ang-2 is typically expressed only at sites of vascular remodeling in the adult (P. C. Maisonpierre, et al., Science 277, 55 (1997)). These expression patterns led to the proposal of a model in which Ang-2 plays a facilitative role at sites of vascular remodeling in the adult by blocking a constitutive stabilizing action of Ang-1 (Figure 1B). Further, it was suggested that such destabilization by Ang-2 in the presence of high VEGF levels primes the vessels to mount a robust angiogenic response reminiscent of that in early embryonic vessels prior to maturation (Figure 1B). However, such destabilization by Ang-2 in the absence of VEGF is instead proposed to lead to frank vessel regression (Figure 1B).

The role of VEGF in tumor-associated angiogenesis apparently recapitulates its actions during normal vascular development. Since VEGF expression is induced in tumor cells by hypoxia (D. Shweiki, M. Neeman, A. Itin, E. Keshet, Proc Natl Acad Sci 92, 768 (1995)), VEGF production is thought to reflect a poorly vascularized tumor, and this VEGF production in turn is thought to contribute to onset of tumor-associated angiogenesis. Consistent with this notion, anti-VEGF

approaches slow the growth of many tumors (K. J. Kim, et al., Nature 362, 841 (1993); B. Millauer, L. K. Shawver, K. H. Plate, W. Risau, A. Ullrich, Nature 367, 576 (1994); R. S. Warren, H. Yuan, M. R. Matli, N. A. Gillett, N. Ferrara, J Clin Invest 95, 1789 (1995); B. Millauer, et al., Cancer Res 56, 1615 (1996); C. K. Goldman, et al., Proc Natl Acad Sci 95, 8795 (1998)). The apparently coordinated actions of VEGF and the Angiopoietins during normal embryonic vascular development, coupled with the suggestion of an important role for Ang-2 during vascular remodeling in normal adult tissues, demanded that the potential actions of the Angiopoietins be examined during tumor angiogenesis.

The lack of improvement in cure rate of many common tumors is amply documented and often ascribed to failure of early detection. Present clinical means for detecting tumor tissue remain in many instances a gross anatomic procedure relying upon various physical findings or radiographic imaging procedures to select a site for histologic sampling. Scintillation imaging techniques with radiopharmaceuticals such as ^{67}Ga-Gallium citrate, ^{111}In-Bleomycin and ^{131}I-Diiodofluorescein have limited success. These radiolabeled compounds lack specificity and sensitivity, that is, they are not preferentially taken up by tumors. Both ^{67}Ga-Gallium citrate and ^{111}In-Bleomycin are accumulated in inflammatory or infectious lesions. Currently, all available diagnostic techniques have many drawbacks and limitations in addition to lack of sensitivity and specificity. These include the use of traumatic invasive procedures and potential for serious complications.

Attempts to "mark" or "tag" tumor cells in order to differentiate them from normal tissues are not new. Various fluorescent compounds such

as porphyrins, tetracycline derivatives, acridine orange and toluidine blue or radioactive isotopes have been extensively investigated. With the exception of porphyrin compounds, none of these substances used by earlier investigators are capable of routinely identifying and delineating tumors and tumor margins.

To be effective, an ideal marker substance should: (1) be safe and non-toxic in humans; (2) selectively accumulate only in tumor tissue and not be taken up by normal or inflammatory tissues; (3) be simple to use and involve non-invasive procedures; (4) be capable of being documented by photographs, radiographs or other recording devices. Unfortunately, the ideal marker or tracer continues to remain elusive.

Technetium-99m (^{99}mTc) based radiopharmaceuticals have been widely used in the past 15 years. They are by far the safest and the most useful scintigraphic imaging agents developed for Nuclear Medicine procedures. The radionuclide ^{99}mTc has many advantages. It is a pure gamma emitter with a relatively short physical half life of six hours. The gamma photon of 140 KeV energy is compatible with existing conventional scintillation imaging equipments. ^{99}mTc-radiopharmaceuticals
can be administered to patients in a much larger dose than many other radiolabeled compounds but produces a minimal radiation health hazard.

For the non-invading nuclear medical diagnosis of tumor, there is ordinarily used gallium citrate ^{67}Ga). While gallium citrate (^{67}Ga) has an accumulating property on tumor cells, it simultaneously possesses the following disadvantages: (1) since its specificity to tumor cells is

low and its energy characteristics are not proper, clear and sharp scintigraphy is hardly obtainable; (2) it takes a long time until the radioactivity disappears from the entire body so that many days are needed for the examination; and (3) its half life is 78.1 hours, and the amount of exposure dose against the patient can not be disregarded. For the above reasons, much research has been done to develop an imaging agent having a high specificity to tumor cells to make a quick diagnosis possible.

One of the recent proposals is imaging of tumor cells using a radioisotope-labeled antibody with a high specificity to tumor cell markers. Since the large scale production of a monoclonal antibody by cell culture of hybridoma cells was reported by Milstein et al. (Nature, Vol. 256, p. 495 (1975)), various antibodies specific to tumor-related antigens have been produced, and imaging of tumor cells using these monoclonal antibodies has been extensively tested. The imaging technique using a radioisotope-labeled antibody is generally called a "radioimmunosintigraphy". Unfortunately, this technique also has inherent problems. For instance, the radioisotope-labeled antibody takes a long time to accumulate on tumor cells and the up-take ratio by these cells is low. Further, the accumulation is done not only by tumor cells, but also by normal organ and tissue cells, and the disappearance of the radioactivity from these organs and tissues takes a long time. For these reasons this technique is has proven impractical.

Studies on the diagnosis of breast cancer have been done with substances specific to steroid hormone receptors such as radioactive iodine-labeled estradiol derivatives (Hanson et al.: American Chemical

Society Meeting, Aug. 3-28, 1981, Reference N.U.S.L. 56; Kabalka: Applications of Nuclear and Radiochemistry, Lambrecht, R. M. Morcosn., Eds., Newark, N.J., Pergamon Press, 1981, Chap. 17; JP-A-60-78995). In order to achieve a reliable diagnosis with these receptor-specific substances, the substances are required to satisfy the following conditions: (1) they have to exhibit high affinity and specificity to the receptor; (2) their specific radioactivity must be sufficiently high; and (3) their labeling nuclide must not be liberated in the body. Unfortunately, radioactive receptor-specific substances satisfying all these conditions have not yet been developed.

Various attempts have been made to identify specific tumor sites by simple techniques. For example, it would be desirable to identify the location of tumor cells by localization of a particular tumor marker at the specific tumor site. It would also be desirable to target the specific tumor site with chemotherapeutic agents by introducing substances into the patient's body that are directed to the tumor marker and that deliver a chemotherapeutic agent to the specific tumor site. In spite of such attempts, however, simple delivery systems for targeting tumors in humans do not as yet exist.

Administering a chemotherapeutic agent usually harms many of the normal body cells, often resulting in a worsening of the patient's condition without achieving the desired reduction in tumor size. Historically, this toxicity to normal cells has been a major disadvantage in the treatment of tumors with chemotherapeutic agents. The lack of efficacy of chemotherapy is also attributed to the failure of

the freely circulating drug to localize within the tumor cells before it is excreted or taken up by other cells in the body.

Prior attempts to improve treatment of tumors by chemotherapeutic agents includes encapsulation of such chemotherapeutic agents within biodegradable phospholipid micellar particles in the form of vesicles or liposomes. Encapsulation is thought to reduce the toxicity caused by the circulating chemotherapeutic agents. Researchers have also sought to utilize encapsulation to selectively target tumors for delivery of chemotherapeutics agents. Unfortunately, efforts to localize or treat tumors with chemotherapeutic agent-encapsulated targeting particles have not been overly successful.

Localization of tumors such as astrocytomas in the brain in vivo and the determination of the margin between normal tissue and tumor can be useful for surgical, radiotherapeutic and chemotherapeutic approaches to treating the tumor. Although gliomas generally do not metastasize, they do recur locally after surgical resection and carry a grave prognosis. The grave prognosis results in part from the inability to delineate clearly the boundary between tumor and normal brain tissue, and from the restricted permeability of the blood brain barrier to imaging and chemotherapeutic agents.

Monoclonal antibodies prepared against tumors have been proposed for use in the past as effective carrier molecules for the delivery of contrast and radionuclide agents. However, the use of such monoclonal antibodies is accompanied by disadvantages. Antibodies are very large molecules that also can carry cross-reactive antigenic determinants

that could cause problems. In addition, the monoclonal antibodies seldom bind more than 70% of cells, even in clonogenic tumors.

In addition to monoclonal antibodies, various synthetic polypeptides, such as polylysine which selectively binds to tumor cells as compared to normal brain cells, have been considered for use as carrier agents for chemotherapeutic agents.

Clearly, a need still exists for reliable, safe methods for the imaging, targeting and treatment of tumors and for substances that can be used in such methods. As Ang-2 appears to be an early marker of blood vessels that have been perturbed by invading tumor cells, Ang-2 may be useful in the imaging of very small tumors and metastases, and may be useful in methods designed to specifically target chemotherapeutic agents to tumor vasculature.

SUMMARY OF THE INVENTION

The subject invention provides for a method for imaging tumor vasculature in a mammal comprising administering to the mammal a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an imaging agent; allowing the composition to accumulate at the tumor vasculature; and detecting the accumulated composition so as to image the tumor vasculature.

The subject invention also provides for a method of causing tumor cell death by targeting tumor vasculature comprising administering to a mammal a composition which comprises a molecule capable of

detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing tumor cell death.

The subject invention further provides for a method of causing vascular endothelial cell death by targeting tumor vasculature comprising administering to a mammal a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing vascular endothelial cell death.

In addition, the subject invention provides for a kit for imaging tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an imaging agent; a kit for targeting tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing tumor cell death; and a kit for targeting tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing vascular endothelial cell death.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-1B. Model for the Coordinated and Complementary Roles of VEGF and the Angiopoietins during Vascular Development and Remodeling, Normally and During Tumorigenesis.

Figure 1A) In the embryo, VEGF is required for differentiation and proliferation of endothelial cells, and for tubule formation. After VEGF promotes the formation of immature vessels, Ang-1, acting through the

Tie2 receptor, promotes the remodeling, maturation and stabilization of blood vessels, which apparently involves optimizing interactions with surrounding support cells and extracellular matrix.

Figure 1B) By adulthood, Tie2 is constitutively engaged with Ang-1, and vessels remain in a stable state. When Ang-2 expression is upregulated, for instance, in the normal ovary or by tumor cells, the interaction between Tie2 and Ang-1 is disrupted, and the vessel is destabilized. Once endothelial cells are separated from perivascular cells and extracellular matrix, they appear particularly plastic and vulnerable. In the presence of VEGF, angiogenesis is promoted; however, lack of stimulatory factors results in the regression of destabilized vessels.

Figure 2A-2N. Vascular status of 1 week, 2 week and 4 week rat gliomas, depicting progressive vessel regression involving dissociation of endothelial and smooth muscle cells and apoptosis.

Figure 2A, Figure 2L. Small one week tumors that measure a fraction of a millimeter in width (T=tumor, scale bar = 1mm (Figure 2A through Figure 2C) or 200μm (Figure 2L through Figure 2N) are well-vascularized as determined by rat endothelial cell antigen (RECA) immunostaining, apparently by co-opting and growing around existing vessels; the vascular density and vessel morphology in early tumors is similar to that of host brain.

Figure 2B, Figure 2M. Two week tumors continue to have extensive internal vasculature, though the vessel density is lower than surrounding brain, presumably due to growth of tumor in the absence of compensatory angiogenesis of existing internal vessels; the caliber of

the internal vessels within these tumors does become dilated and relatively uniform compared to normal brain vessels.

Figure 2C, Figure 2N. Within large four week tumors, internal vessels regress with accompanying loss of surrounding tumor (necrotic tumor areas are unstained). Surviving internal vessels are sparse and uniform, centrally located with respect to surrounding cuffs of well-stained viable tumor cells, and exhibit no evidence of compensatory angiogenesis, though robust angiogenesis is apparent at margin of the tumor, where increase density of ectatic vessels is noted. Arrowheads depict a patent (top) and a regressed (bottom) vessel each surrounded by either surviving regressed cuff of tumor.

Figure 2D through Figure 2G. Vessel-specific apoptosis (S. D. Morgenbesser, et al., EMBO J 14, 743 (1995).) is specifically noted in tumors (Figure 2D through Figure 2F) prior to widespread apoptosis in tumor cells themselves (Figure 2G); arrowheads denote vessel-specific apoptotic figures (stained black) in Figure 2D through Figure 2G.

Figure 2H through Figure 2K. Immunostaining with antibodies to SMA (black) in combination with those to RECA (brown) show that pericytes and smooth muscle cells detach from the vessel wall in tumors. Figure 2H shows a vessel wall in normal brain in which RECA and SMA staining are essentially superimposed, whereas Figure 2I through Figure 2K depict vessels within tumors with varying stages of detachment of SMA positive cells. Scale bar in Figure 2D = 50 μm (Figure 2D through Figure 2K).

Figure 3A-Figure 3L. In situ hybridization analysis of Ang-2, Tie and VEGF messages in two slightly different 2 week rat glioma tumors, as well as in large 4 week rat glioma. In two week tumors, the vessels

within both a small (earlier stage) tumor (Figure 3A through Figure 3D) and a large (slightly later stage) tumor (Figure 3E through Figure 3G) consistently express high levels of Ang-2 (Figure 3C, Figure 3G). In contrast, upregulation of Tie message (Figure 3A, Figure 3E) is restricted to the later stage tumor. Induction of VEGF is very minimal in early stage tumors (Figure 3B) and still modest and patchy in later stage tumors (Figure 3F). In large 4 week tumors, the tumor is secondarily avascular due to massive vessel regression and thus has few internal vessels, but has a hypervascular plexus at the tumor border. Both the few internal and the many rim vessels are now emphatically marked by both Ang-2 and Tie (Figure 3I, Figure 3K), though expression of Ang-2 is more punctate than that of Tie, and the remaining live tumor cuffs around vessels have dramatically upregulated VEGF expression (Figure 3J). This VEGF expression is highest in palisading, presumably hypoxic tumor cells which are furthest from vessels; large areas within the tumor, between palisading cells, are necrotic. Figure 3D, Figure 3H and Figure 3L outline the boundaries of the tumor within the brain and indicate the relative levels of expression of Ang-2, Tie and VEGF. Scale bar in Figure 3A is 500μm for Figure 3A through Figure 3H, and scale bar in Figure 3I = 1mm for Figure 3I through Figure 3L.

Figure 4A-Figure 4F. Human Glioblastoma Shows Upregulation of Angiopoietin-2 Specifically in Tumor Vasculature. Figure 4A shows an overview of an azocarmine stained human glioblastoma (tumor) including its advancing invading edge (inv). The tumor includes a necrotic area (nec) and a viable periphery. Based on the morphological appearance of the blood vessels, four zones can be defined. Zone 1 is

adjacent to the necrotic zone (nec) and displays hyalinized vessels (black arrows Figure 4A and Figure 4B). Zone 2 has well perfused ectatic vascular channels (black arrowheads Figure 4A and Figure 4B). Between zones 1 and 2 are partially hyalinized channels (*). Zone 3 displays vascular channels with early hyperplasia (white arrows). Zone 4 contains morphologically quiescent appearing normal vessels not yet invaded by tumor (white arrowheads). Inset in Figure 4B is enlargement of tumor area delineated by box in Figure 4A. Figure 4C through Figure 4F show *in situ* hybridization analysis of glioblastoma using a digoxigenin probe. In Zone 4 (Figure 4C) there is no Ang-2 signal in vessels of the normal brain or in microvessels distant from the tumor "margin". An unlabeled vascular channel is seen (v). High Ang-2 expression (black digoxigenin-based stain) is apparent in vessels in all other zones. This includes Zone 3 (Figure 4D) which contains vascular channels without hyperplastic changes, as well as the hyperplastic vessels in Zone 2 (Figure 4E) and the sclerotic vessels in Zone 1 (in which positive endothelial cells are denoted with a white arrow) (Figure 4F). Scale bar = 200μm in Figure 4A; 100μm in Figure 4B and Figure 4C; 50 μm in Figure 4D and 25μm in Figure 4E and Figure 4F.

Figure 5A-Figure 5D. *In situ* hybridization analysis of Ang-2 transcripts in rat RBA mammary carcinomas and mouse Lewis Lung Carcinomas shows upregulation in co-opted tumor vessels.

(Figure 5A) Section through a mammary carcinoma stained with cresyl violet demonstrates the invasiveness of the tumor cells in the brain. The inset shows a similar section immunostained with antibodies to RECA; the tumor cells form small islands (stained orange/brown) around co-opted blood vessels (stained black).

(Figure 5B) While VEGF is typically weak or undetectable at this tumor stage (not shown), Ang-2 is highly expressed in a punctate manner by blood vessels. Scale bar in Figure 5A = 200 μm (Figure 5A, Figure 5B) or 400 μm (inset).

(Figure 5C) Section through a Lewis Lung carcinoma stained with cresyl violet demonstrates a tiny metastasis (left, arrowheads) and a still small metastasis (right, arrows); vessels, stained black, lie within these small tumors.

(Figure 5D) The vessels co-opted by the small Lewis Lung metastases exhibit dramatic induction of Ang-2. Scale bar in Figure 5C = 500 μm (Figure 5C, Figure 5D).

DETAILED DESCRIPTION OF THE INVENTION

The subject invention provides for a method for imaging tumor vasculature in a mammal comprising administering to the mammal a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an imaging agent; allowing the composition to accumulate at the tumor vasculature; and detecting the accumulated composition so as to image the tumor vasculature.

The subject invention also provides for a method of causing tumor cell death by targeting tumor vasculature comprising administering to a mammal a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing tumor cell death.

The subject invention further provides for a method of causing vascular endothelial cell death by targeting tumor vasculature comprising administering to a mammal a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing vascular endothelial cell death.

In addition, the subject invention provides for a kit for imaging tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an imaging agent; a kit for targeting tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing tumor cell death; and a kit for targeting tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing vascular endothelial cell death.

Specific embodiments of the invention include a molecule capable of detecting Ang-2 nucleic acid wherein the molecule is a nucleic acid, a mRNA, a synthetic oligonucleotide.

Specific embodiments of the invention also include a molecule capable of detecting Ang-2 polypeptide wherein the molecule is a polypeptide, a synthetic polypeptide, a monoclonal antibody, an antibody fragment, a single chain fv, a Tie 1-Fc receptorbody polypeptide, a Tie 2-Fc receptorbody polypeptide, a Tie 1 receptor fragment polypeptide containing an Ang-2 binding domain, or a Tie 2 receptor fragment polypeptide containing an Ang-2 binding domain.

The subject invention provides for methods of detecting an imaging agent using, for example, a conventional scintillation camera, a gamma camera, a rectilinear scanner, a PET scanner, a SPECT scanner, a MRI scanner, a NMR scanner, or an X-ray machine.

The subject invention also provides for imaging agents which are radionuclides or chelates.

The subject invention further provides for agents capable of causing tumor cell death and vascular endothelial cell death.

Our analyses in several different tumor settings challenge the prevailing view that malignancies and metastases generally initiate as avascular masses that only belatedly induce vascular support. Instead, we find that malignant cells rapidly co-opt existing host vessels to form an initially well-vascularized tumor mass. Paradoxically, the co-opted vasculature does not undergo angiogenesis to support the growing tumor, but instead regresses (perhaps as part of a normal host defense mechanism) via a process that involves disruption of endothelial cell/smooth muscle cell interactions and endothelial cell apoptosis. This vessel regression in turn results in necrosis within the central part of the tumor. However, robust angiogenesis is initiated at the tumor margin, supporting further growth. The expression patterns of Ang-2 and vascular endothelial growth factor (VEGF), strongly implicate these factors in the above processes. Ang-2 is highly expressed in co-opted vessels, prior to VEGF induction in the surrounding tumor cells, providing one of the earliest markers of tumor vasculature and apparently marking the co-opted vessels for

regression. Subsequently, VEGF upregulation coincident with Ang-2 expression at the tumor periphery is associated with robust angiogenesis. Thus, in tumors, Ang-2 and VEGF seem to reprise the roles they play during vascular remodeling in normal tissues, acting to regulate the previously under-appreciated balance between vasculature regression and growth.

As Ang-2 appears to be an early marker of tumor vascularization, Applicant reasoned that imaging Ang-2 would be an extremely useful technique for identifying tumors at an early stage and, once identified, Ang-2 could be used as a target for delivering chemotherapeutic agents to the tumor site. By targeting Ang-2 for delivery of such an agent, one could achieve high local concentrations of the chemotherapeutic agent at the tumor site, while at the same time minimizing non-specific accumulation of the agent at non-tumor sites. Because Ang-2 is highly expressed early in tumor vascularization, two approaches can be used to deliver the chemotherapeutic agent to the tumor site. In one embodiment of the invention, Ang-2 can be coupled to any chemotherapeutic agent capable of causing tumor cell death. Non-limiting examples of chemotherapeutic agents that are suitable for coupling to Ang-2 include carboplatin, cisplatin and other related platinum-based agents; vincristine; methotrexate; taxanes such as paclitaxel and docetaxel; fluorinated pyrimidines such as 5-fluorouracil and UFT (tegafur and uracil); hydroxyurea; gemcitabine; vinorelbine; irinotecan; tirapazamine; and matrilysin.

In an alternate embodiment of the invention, Applicant reasoned that because Ang-2 is highly expressed in tumor vasculature by the vascular

endothelial cells, it is possible to deliver an agent capable of causing death of the vascular endothelial cells by coupling the agent to Ang-2. Vascular endothelial cell death will necessarily result in vessel regression which will ultimately lead to tumor cell death due to a lack of nutrient supply. Non-limiting examples of agents capable of causing vascular endothelial cell death that would be suitable for coupling to Ang-2 include gelonin, ricin A, ricin B, saporin, bryodin 1, bryodin 2, momordin, pokeweed antiviral protein from seeds (PAP-S), trichokirin, and abrin.

To image or target Ang-2 in tumor vasculature, it is necessary to administer a composition comprising a molecule capable of detecting Ang-2. Several non-limiting examples of molecules that would be suitable for detecting Ang-2 include Tie 1-Fc receptorbody polypeptide molecules and Tie 2-Fc receptorbody polypeptide molecules; Tie 1 receptor fragment polypeptide molecules containing an Ang-2 binding domain and Tie 2 receptor fragment polypeptide molecules containing an Ang-2 binding domain; anti-Ang-2 monoclonal antibodies, anti-Ang-2 antibody fragments, anti-Ang-2 single chain fvs; and nucleic acids including, but not limited to, mRNAs and synthetic oligonucleotides. In fact, any molecule capable of specifically binding to or associating with Ang-2 would a suitable detecting molecule.

For preparation of monoclonal antibodies directed toward Ang-2, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human

B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy", Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies for imaging or targeting use may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (e.g., Teng et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:7308-7312; Kozbor et al., 1983, Immunology Today 4:72-79; Olsson et al., 1982, Meth. Enzymol. 92:3-16). Chimeric antibody molecules may be prepared containing a mouse antigen-binding domain with human constant regions (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851, Takeda et al., 1985, Nature 314:452).

In addition to monoclonal antibodies, the subject application provides for fragments of such monoclonal antibodies. Antibody fragments which contain the idiotype of the antibody can be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent. Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity

chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof.

In addition to monoclonal antibodies and fragments of such monoclonal antibodies, the subject application provides for single chain Fvs (scFv). A scfv is a truncated Fab having only the V region of a heavy chain linked by a stretch of synthetic peptide to a V region of a light chain. See, for example, US Patent Nos. 5,565,332; 5,733,743; 5,837,242; 5,858,657; and 5,871,907 assigned to Cambridge Antibody Technology Limited incorporated by reference herein.

Other suitable agents able to detect Ang-2 are Tie 1 and Tie 2 receptorbody polypeptides. Tie 1 and Tie 2 receptorbody polypeptides are secreted protein consisting of the entire extracellular portion of the Tie 1- or Tie 2 receptor fused to the human immunoglobulin gamma-1 constant region (IgG1 Fc). These fusion protein would be normally expected to exist as dimers in solution based on formation of disulfide linkages between individual IgG1 Fc tails. For a detailed description of how to make Tie receptorbody polypeptides, see published PCT application WO 96/11269, published April 18, 1996 or published PCT application WO 96/31598, published October 10, 1996, both of which are incorporated by reference in their entireties.

Parenteral administration includes, but is not limited to, intramuscular, intravenous, subcutaneous, intrathecal, and intraperitoneal injection.

Suitable imaging agents that can be coupled to Ang-2 include, but are not limited to, agents useful in magnetic resonance imaging (MRI) such as gadolinium chelates (see for example Ladd, DL, et al., 1999, Bioconjug Chem 10:361-370), covalently linked nonionic, macrocyclic, multimeric lanthanide chelates (see for example Ranganathan, RS, et al., 1998, Invet Radiol 33:779-797), and monoclonal antibody-coated magnetite particles (see To, SY, et al., 1992, J Clin Laser Med Surg 10:159-169). For reviews relating to basic principles of MRI see Kirsch, JE, 1991, Top Magn Reson Imaging 3:1-18 and Wallis, F and Gilbert, FJ, 1999, J R Coll Surg Edinb 44:117-125. Radionuclides are also suitable imaging agents for use in nuclear medicine techniques such as positron emission tomography (PET), single positron emission computed tomography (SPECT), and computerized axial tomography (CAT) scans. By way of non-limiting example, such agents include technetium 99m, gallium 67 citrate, iodine 123 and indium 111 (see Coleman, RE, 1991, Cancer 67:1261-1270). Other radionuclides suitable as imaging agents include ^{123}I and ^{111}In-DTPA (see Kaltsas, GA, et al., 1998, Clin Endocrinol (Oxf) 49:685-689), radiolabeled antibodies (see Goldenberg, DM and Nabi, HA, 1999, Semin Nucl Med 29:41-48 and Steffens, MG, et al., 1999, J Nucl Med 40:829-836). For reviews relating to basic principles of radionuclear medicine techniques, see Schiepers, C. And Hoh, CK, 1998, Eur Radiol 8:1481-1494 and Ferrand, SK, et al., 1999, Surg Oncol Clin N Am 8:185-204.

Before the present methods and kits for making and using the invention are described, it is to be understood that the invention is not to be limited only to the particular methods or kits described. The method and kits may vary, and the terminology used herein is for the purpose of

describing particular embodiments. The terminology and definitions are not intended to be limiting since the scope of protection will ultimately depend upon the claims.

EXAMPLES

Materials and Methods

(a) Cell Culture and Tumor Implantation

C6 glioma, RBA mammary carcinoma and Lewis Lung carcinoma cells were obtained from ATCC and grown in culture. ~1.0 X 10^5 C6 or RBA cells were suspended in ~2µl of phosphate buffered saline and injected stereotaxically over a period of 5 to 10 minutes into the right striatum (AP + 0.5; ML -3.0; DV -6.0 relative to Bregma) of adult male Sprague Dawley rats. Approximately 5.0 X 10^5 Lewis Lung carcinoma cells were suspended in 50µl of serum free media and injected into the jugular vein of adult male C57Bl mice.

(b) Tissue Preparation and Immunocytochemistry

Rats and mice were anesthetized and decapitated or perfused with 4% paraformaldehyde. Brains for thick sections were post-fixed in 4% paraformaldehyde overnight and then equilibrated in 35% sucrose. Forty µm thick sections were cut on a sliding microtome. For thin sections (10µm), fixed brains were equilibrated in 17% sucrose. Fresh and fixed brains were frozen in methylbutane chilled in dry ice. Cryostat sections were collected on ProbeOn or Superfrost Plus slides

(Fischer). For TUNEL labeling (S. D. Morgenbesser, et al., EMBO J 14, 743 (1995)) brains were immersion fixed in 10% neutral buffered formalin and embedded in paraffin.

Sections from perfused rats were immunostained with a monoclonal antibody to rat endothelial cell antigen (RECA1; Serotec; 1:250) and a biotinylated horse anti-mouse secondary antibody (1:1500; Vector) using methods as previously described (J. K. Morse, et al., J Neurosci 13, 4146 (1993)). For double labeling a similar protocol was employed. Sections were initially labeled with a monoclonal antibody to alpha smooth muscle actin (SMA; DAKO; 1:500) and a biotinylated goat anti-mouse IgG IIa secondary antibody (1:1250; Amersham). After visualizing staining with a Vectastain elite kit (Vector) and producing a black reaction product by using nickel sulfate enhancement, sections were then blocked again and labeled with the antibody to RECA (1:100). A brown reaction product was used.

(c) Rat and Mouse Tumor *In Situ* Hybridization

Either fresh frozen or fixed sections were used for *in situ* hybridization. Sections were probed with ^{35}S-labeled cRNAs as described (D. M. Valenzuela, et al., Neuron 10, 963 (1993)). Probes for VEGF, Ang-1 and Ang-2 have previously been described (P. C. Maisonpierre, et al., Science 277, 55 (1997)). For Tie1, a 1.3 kb fragment of rat Tie1 spanning the last 309 codons and 375 bp of the 3' untranslated sequence was used and for Tie2, a 460 bp fragment spanning codons 771 to 924 within the kinase domain was used.

(d) Human Glioblastoma *In Situ* Hybridization

Five glioblastomas multiforme (GBMs) diagnosed according to the WHO grading system all showed focal areas of necrosis with or without pseudopalisading and contained hyperplastic blood vessels which often had a glomeruloid appearance. Specimens were treated as previously described.

Example 1: Characterization of C6 Tumor Model and its Associated Vasculature: Co-opting of host vessels at early stages, followed by vessel regression

The first tumor model examined was a well-characterized rat model of glioblastoma (N. Nagano, H. Sasaki, M. Aoyagi, K. Hirakawa, Acta Neuropathol. 86, 117 (1993)), in which malignant rat C6 glioma cells are injected stereotaxically into rat brain. In order to perform a careful temporal assessment of VEGF and Angiopoietin expression patterns during tumor progression, we first characterized this model with respect to its vasculature, particularly in the very early stages of tumor development. The tumors and their associated vasculature were evaluated at early (1 week), intermediate (2 week), and advanced (4 week) stages of tumor development; endothelial cells within the tumor and adjacent brain were stained using a monoclonal antibody to rat endothelial cell antigen (RECA-1; (A. M. Duijvestin, et al., Lab Invest 66, 459 (1992)).

Remarkably, even the smallest tumors, measuring just a fraction of a millimeter in width, are well vascularized (Figure 2A, Figure 2L). As

previously noted (N. Nagano, H. Sasaki, M. Aoyagi, K. Hirakawa, Acta Neuropathol. 86, 117 (1993)), this is attributable to the co-opting of existing brain blood vessels by the implanted tumor cells. Consistent with this, the vascular density of these early tumors is similar to that of host brain, and the structural heterogeneity of the vessels within the tumors also resembles that of the surrounding normal brain. There is no evidence of angiogenesis at this early one week time point. By two weeks post-implantation, the tumors have grown to two or more millimeter in diameter (Figure 2B, Figure 2M). Vessel density is decreased within the tumors, but there is no obvious angiogenic response (Figure 2B, Figure 2M). However, vessels within these tumors are distinctly larger and more homogeneous in caliber than the microvasculature of the normal brain. Late stage tumors (4 weeks after implantation), measure several millimeters in size, and have undergone remarkable changes when compared to smaller tumors at earlier stages of development (Figure 2C, Figure 2N). Blood vessels within the core of the tumor have undergone dramatic regression, with no evidence of a local, compensatory angiogenic response. Thus the centers of the tumors are largely bereft of vessels, leading to massive tumor cell death (Figure 2C, Figure 2N). The remaining live tumor cells are organized in cuffs of pseudopalisading cells around the few surviving internal vessels (Figure 2C, Figure 2N). In contrast to the lack of an angiogenic response within the interior of the tumor, a robust angiogenic response is obvious at the rim of these large tumors, as evidenced by the development of a dense plexus of ectatic vessels (Figure 2C, Figure 2N).

Analysis of the early C6 tumors confirms that they do not initially grow as avascular masses, but instead immediately home in on and co-opt existing host vessels. These co-opted vessels do not immediately respond to the growing tumor by undergoing angiogenesis, but instead regress. This vessel regression leads to the demise of the dependent tumor cells. However, the tumor appears to be 'rescued' by the onset of angiogenesis at the periphery of the tumor mass.

Example 2: Regression of Early Tumor Vasculature is Associated with Endothelial Cell Apoptosis and Changes in Endothelial Cell/Smooth Muscle Interactions

To confirm the notion that blood vessel regression is an early event which precedes widespread tumor cell death, we performed *in situ* analysis of apoptosis during tumor progression. In well-vascularized tumors apoptosis is first detected within the co-opted blood vessels, particularly in endothelial cells, with little evidence of cell death in the surrounding tumor (Figure 2D through Figure 2F); such vessel-associated apoptosis is rarely noted in normal brain tissue. At later stages of tumor growth, apoptosis is rampant throughout the tumor mass (Figure 2G).

To further explore this seemingly paradoxical regression of the initially co-opted vessels (Figure 2C), we double-stained tissue sections using both RECA and smooth muscle actin (SMA), a marker of smooth muscle cells and pericytes, to determine whether changes in the integrity of the cellular investment of the vessels was occurring as tumor development progressed. Although many vessels in normal brain

and early tumors are not labeled with SMA, when apparent, SMA positive cells closely invest endothelial cells (Figure 2H). As tumor growth progresses, more and more examples are noted of SMA-positive cells which are partially or completely disengaged from endothelial cells (Figure 2I); such disengagement is never noted in normal brain tissue. The detection of such disengaged structures is followed by the appearance of clearly regressed vascular elements marked by scattered debris of both cell types (Figure 2J, Figure 2K).

Example 3: *In situ* Hybridization Analysis Reveals Ang-2 to be Earliest Marker of Tumor Vessels

The apparent association of tumor vessel regression and apoptosis with a disruption in the interactions between endothelial cells and their smooth muscle investiture was quite striking, and of particular interest to those studying the roles of the Angiopoietins. Mice engineered to lack Ang-1, or to transgenically over-express the antagonist Ang-2, display defects in endothelial cell/smooth muscle interactions associated with vascular anomalies and blood vessel regression (Figure 1A), reminiscent of the changes noted above in tumor-associated vessels. Thus temporal examination of Angiopoietin expression in these tumors was a requisite next step.

To explore the relationship between the temporal changes in tumor vasculature and the expression of VEGF and Angiopoietins, we performed *in situ* hybridization on tumors at various stages of development; probes were used for VEGF, Ang-1 and Ang-2, as well as Tie1 and 2. The most striking observations involved Ang-2 expression,

which we found to be an early and specific marker of both existing vessels that have been co-opted by the implanted tumor cells and are destined to regress, as well as vessels in later tumors undergoing robust angiogenesis.

In small two week tumors (Figure 3A through Figure 3D), the patterns of Tie1 or Tie2 expression do not readily distinguish tumor associated vessels from those outside the tumor, as vessels in normal brain express these receptors at low to moderate levels. Similarly, VEGF is not expressed within the vast majority of tumor cells, presumably because they are reasonably well vascularized at this point and not hypoxic. However, the initially co-opted tumor vessels are extraordinarily well-marked by Ang-2 expression, which clearly distinguishes these vessels from those in surrounding normal brain which do not express detectable Ang-2. Ang-2 marks most but not all of the co-opted vessels, and expression was noted to be by endothelial cells themselves.

In slightly larger tumors (Figure 3E through Figure 3H), Ang-2 dramatically marks almost all tumor associated vessels, and upregulation of the expression of the Tie receptors is also apparent. Additionally, a weak and inconsistent upregulation of VEGF expression is noted in the tumor cells themselves. The variability of VEGF mRNA expression is apparent both within a single tumor and among tumors, likely reflecting the initial stages of regional hypoxia within the growing tumors. The variability of VEGF expression contrasts sharply with the expression of Ang-2, which is consistently upregulated in vessels throughout the tumor.

In larger tumors, further changes in expression patterns are obvious (Figure 3I through Figure 3L). Expression of Ang-2 continues to emphatically mark the few surviving internal vessels, as well as the actively angiogenic vessels at the tumor periphery (Figure 3L). Tie receptors are similarly up-regulated in vessels at the tumor margin (Figure 3I). Strikingly, VEGF expression is now dramatically elevated within much of the remaining, and presumably hypoxic, tumor (Figure 3J). VEGF expression is particular strong at the periphery of the tumor, immediately deep to the rim of ectatic vessels, as well as in cuffs of tumor cells which surround the few remaining internal vessels.

Throughout tumor development, Ang-1 expression is not particularly striking. Consistent with observations that C6 cells express low levels of Ang-1 in culture (B. Enholm, et al., Oncogene 14, 2475 (1997)), Ang-1 expression was noted diffusely in tumors at one week post-implantation. In contrast to the marked elevation of VEGF in hypoxic regions of large tumors, Ang-1 expression was not notably elevated consistent with *in vitro* studies showing that, unlike VEGF, Ang-1 is not upregulated by hypoxia (B. Enholm, et al., Oncogene 14, 2475 (1997))

The various changes in expression patterns noted by *in situ* hybridization, including the marked early induction of Ang-2 expression, were confirmed by Northern analysis.

Example 4: Findings in Human Glioblastomas are Consistent with those in the Rat Model

Advanced experimental C6 glioblastomas in the rat share many histological features with human glioblastomas. The center of human glioblastomas is typically necrotic and largely lacks patent blood vessels (Figure 4A, "nec" area), while the periphery of the tumor is marked by a high density of ectatic and hyperplastic vessels with enlarged and irregular lumens (Figure 4A, Zone 2 and 3). These are easily distinguished from the quiescent vessels found in normal brain (Figure 4A, Zone 4). The transitional zone between the central area of necrosis and the tumor rim contains regressing and hyalinized vessels (Figure 4A, Zone 1), while the transitional zone between tumor rim and normal brain contains vessels that exhibit early hyperplastic changes (Figure 4A, Zone 3). This static view of human glioblastoma reveals a progression of vascular changes that resemble those seen during tumor growth in the rat model, characterized by regression of internal vessels as the tumor continues to infiltrate and induce angiogenesis at its periphery. *In situ* hybridization analysis human glioblastoma also reveals patterns of Angiopoietin expression which are strikingly analogous to those seen in the rat model. That is, quiescent vessels in normal brain do not express Ang-2 (Figure 4C), whereas vessels which exhibit very early hyperplastic changes in the transitional Zone 3 show dramatic upregulation of Ang-2 (Figure 4D). High levels of Ang-2 expression are also evident in the obviously hyperplastic vessels in Zones 2 and 3 (Figure 4E) as well as in regressing vessels within Zone 4 (Figure 4F). Thus, as in the rat model, Ang-2 expression emphatically marks vessels undergoing change in human glioblastomas, whether it be

hyperplastic or regressive; and Ang-2 expression is once again localized to endothelial cells themselves. Ang-1 expression also resembles that seen in the rat model, being characterized by diffuse expression in tumor cells, with little evidence of upregulation in presumably hypoxic areas.

More detailed analysis of human tumors confirms other features of our rat model. For example, early regressing vessels are marked by disruption in endothelial cell/smooth muscle interactions as well as by apoptosis of endothelial cells prior to the apoptosis of surrounding tumor cells.

Example 5: Other Tumor Models

The analyses described above demonstrate that Ang-2 expression is an early and specific marker of vessels undergoing either regressive or angiogenic changes in both rat and human glioblastomas. To begin to explore the possibility that these findings are generalizable to other types of tumors, we examined an unrelated tumor cell line, the RBA mammary adenocarcinoma, implanted stereotaxically into the brain. As for C6 and carcinoma cells (D. Zagzag, S. Brem, F. Robert, Am J Pathol 13, 361 (1988)), implanted RBA cells do not grow initially as an avascular mass, but rapidly associate with, and migrate along cerebral blood vessels in a manner that is even more striking than that observed in gliomas (Figure 5A). Consistent with the well-vascularized state of these tumors, there is minimal upregulation of VEGF. However, the co-opted vessels once again display striking and specific upregulation of Ang-2, which is not detected in the vessels of neighboring normal brain

(Figure 5B). Ang-1 is not notably expressed in RBA-derived tumors, consistent with the lack of expression of Ang-1 by RBA cells in culture. Furthermore, preliminary analysis of later stages of RBA tumor progression indicate that Ang-2 expression is associated with a pattern of vascular regression and angiogenesis which resembles that evident in glioblastomas.

Examination of a model of tumor metastasis, in which mouse lung is colonized by intravenously injected Lewis Lung Carcinoma cells, once again yields similar results. Tiny tumor metastases (arrowheads, Figure 5C, Figure 5D) as well as moderately-sized tumor nodules (arrows, Figure 5C, Figure 5D) are closely associated with pulmonary vessels, and these vessels display dramatic induction of Ang-2 expression (Figure 5D). Progressively larger tumor nodules appear to be characterized by vessel regression as well as new-onset angiogenesis, again co-incident with high levels of expression of Ang-2.

The generality of our findings is further demonstrated by experiments in which rat C6 glioma cells are implanted either subcutaneously or into the lung (via the vasculature) of mice. In both instances the rat tumor cells are able to induce upregulation of Ang-2 in the host vasculature as well as new vessels which have formed within the growing tumor mass. Similarly, when human HT-1080 fibrosarcoma cells are implanted subcutaneously into the mouse, they too induce upregulation of Ang-2 in associated vessels.

Preliminary analysis of additional tumor models, including transgenic mouse models of tumor development further support the general

relevance of our findings: Ang-2 is upregulated in diverse types of solid tumors, irrespective of tumor location, in both extant blood vessels coopted by the tumors and new blood vessels generated in the course of tumor growth. This also appears to be the case in humans, as our initial evaluations show that Ang-2 is upregulated, non only in glioblastoma, but also in colon and breast carcinoma. Thus, Ang-2 may is a very general marker of tumor vessels undergoing either regressive or angiogenic changes. Furthermore Ang-2 may play a critical early role in initiating these processes, as has been previously suggested in the context of the cyclic vascular remodeling of the female reproductive system (Figure 1B).

Discussion

It has been widely suggested that tumors and metastases originate as small avascular structures that are limited in their ability to grow beyond a few millimeters in size, unless they induce an angiogenic response from surrounding host vessels. However, some prior evidence suggests that many tumors may not grow initially in an avascular fashion in the absence of angiogenesis, but may instead coopt existing blood vessels (D. Hanahan and J. Folkman, Cell 86, 353-364 (1996)). Our temporal and spatial examination of the early stages of tumor growth in several different tumor settings is consistent with the notion that many solid tumors do not initially grow avascularly, but instead home to and co-opt existing parenchymal vessels. Surprisingly, we have found that an angiogenic response is not immediately stimulated in blood vessels co-opted by the tumor. Rather, the co-opted vessels paradoxically regress, leading to the demise of surrounding tumor cells.

While classic tumor studies have noted the apparent collapse and regression of internal tumor vessels, they often attribute this to high interstitial pressures within tumors; however, recent analyses make such explanations unlikely and necessitate alternate explanations for the vessel loss (Y. Boucher, R. K. Jain, Cancer Res. 52, 5110 (1992)). We suggest that vessel regression and associated tumor loss may be part of a defense mechanism which occurs in response to the disruption of the normal vascular milieu. Despite this response, the successful tumor is ultimately not constrained by the regression of vessels at the tumor core, as a robust angiogenic response is initiated at the tumor margin, where malignant cells continue to infiltrate host tissues. However, once formed, tumor vessels appear to be unstable and continue to regress, such that tumors may have to invade deeper into adjacent normal tissues if they are to survive.

Our detailed expression studies point towards key roles in the above processes for members of the two known families of vascular endothelium-specific growth factors. Vessel regression occurs early on when Ang-2 is expressed in the absence of VEGF, whereas robust angiogenesis is subsequently noted at sites of coincident Ang-2 and VEGF expression (Figure 1B). Similar expression patterns have been noted during the initial stages of normal vascular remodeling in the female reproductive system (P. C. Maisonpierre, et al., Science 277, 55 (1997)), suggesting that these factors may serve generally important roles at all sites of vascular change.

The precise signal which leads to the dramatic and specific upregulation of Ang-2 in vessels co-opted by tumor cells remains

unknown. However, the autocrine upregulation of Ang-2 presumably reflects a response by affected endothelial cells to tumor-associated perturbations in the perivascular milieu. The upregulation of Ang-2 in tumor associated vessels occurs before VEGF expression is obviously elevated, and at a point when small tumors or metastases appear to be adequately vascularized.

Ang-2 appears to be an early marker of blood vessels that have been perturbed by invading tumor cells. As such, Ang-2 may prove to be useful in the imaging of very small tumors and metastases, and may even be useful in methods designed to specifically target chemotoxic therapy to tumor vasculature.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WE CLAIM

1. A method for imaging tumor vasculature in a mammal comprising a) administering to the mammal a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an imaging agent;
b) allowing the composition to accumulate at the tumor vasculature; and
c) detecting the accumulated composition so as to image the tumor vasculature.

2. The method of claim 1 wherein the Ang-2 and the molecule capable of detecting Ang-2 are nucleic acids.

3. The method of claim 1 wherein the Ang-2 and the molecule capable of detecting Ang-2 are polypeptides.

4. The method of claim 1 wherein the accumulated composition is detected by a detector selected from the group consisting of a conventional scintillation camera, a gamma camera, a rectilinear scanner, a PET scanner, a SPECT scanner, a MRI scanner, a NMR scanner, and an X-ray machine.

5. The method of claim 1 wherein the imaging agent is a radionuclide or a chelate.

6. A method of causing tumor cell death by targeting tumor vasculature comprising administering to a mammal a composition

which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing tumor cell death.

7. A method of causing vascular endothelial cell death by targeting tumor vasculature comprising administering to a mammal a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing vascular endothelial cell death.

8. The method of claim 6 wherein the Ang-2 and the molecule capable of detecting Ang-2 are nucleic acids.

9. The method of claim 7 wherein the Ang-2 and the molecule capable of detecting Ang-2 are nucleic acids.

10. The method of claim 6 wherein the agent capable of causing tumor cell death is selected from the group consisting of carboplatin, cisplatin, vincristine, methotrexate, paclitaxel, docetaxel, 5-fluorouracil, UFT, hydroxyurea, gemcitabine, vinorelbine, irinotecan, tirapazamine, and matrilysin.

11. The method of claim 6 wherein the Ang-2 and the molecule capable of detecting Ang-2 are polypeptides.

12. The method of claim 7 wherein the Ang-2 and the molecule capable of detecting Ang-2 are polypeptides.

13. The method of claim 7 wherein the agent capable of causing vascular endothelial cell death is selected from the group consisting of gelonin, ricin A, ricin B, saporin, bryodin 1, bryodin 2, momordin, pokeweed antiviral protein from seeds (PAP-S), trichokirin, and abrin.

14. The method of claim 1, 6, or 7 wherein the mammal is a human.

15. The method of claim 1, 6, or 7 wherein the molecule capable of detecting Ang-2 polypeptide is selected from the group consisting of a monoclonal antibody, an antibody fragment, and a single chain fv.

16. The method of claim 1, 6, or 7 wherein the molecule capable of detecting Ang-2 polypeptide is selected from the group consisting of a Tie 1-Fc receptorbody polypeptide, a Tie 2-Fc receptorbody polypeptide, a Tie 1 receptor fragment polypeptide containing an Ang-2 binding domain, and a Tie 2 receptor fragment polypeptide containing an Ang-2 binding domain.

17. The method of claim 1, 6, or 7 wherein the composition is administered to a mammal with a carrier suitable for parenteral administration.

18. The method of claim 17 wherein the mammal is a human.

19. The method of claim 2, 8, or 9 wherein the molecule capable of detecting Ang-2 nucleic acid is an mRNA.

20. The method of claim 2, 8, or 9 wherein the molecule capable of detecting Ang-2 nucleic acid is a synthetic oligonucleotide.

21. The method of claim 3, 11, or 12 wherein the molecule capable of detecting Ang-2 polypeptide is a synthetic polypeptide.

22. A kit for imaging tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an imaging agent.

23. The kit of claim 22 wherein the Ang-2 and the molecule capable of detecting Ang-2 are nucleic acids.

24. The kit of claim 22 wherein the Ang-2 and the molecule capable of detecting Ang-2 are polypeptides.

25. A kit for targeting tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing tumor cell death.

26. A kit for targeting tumor vasculature in a mammal comprising a composition which comprises a molecule capable of detecting Ang-2 nucleic acid or polypeptide coupled to an agent capable of causing vascular endothelial cell death.

27. The kit of claim 25 wherein the Ang-2 and the molecule capable of detecting Ang-2 are nucleic acids.

28. The kit of claim 26 wherein the Ang-2 and the molecule capable of detecting Ang-2 are nucleic acids.

29. The kit of claim 25 wherein the Ang-2 and the molecule capable of detecting Ang-2 are polypeptides.

30. The kit of claim 26 wherein the Ang-2 and the molecule capable of detecting Ang-2 are polypeptides.

31. The kit of claim 22, 25, or 26 wherein the molecule capable of detecting Ang-2 polypeptide is selected from the group consisting of a monoclonal antibody, an antibody fragment, and a single chain fv.

32. The kit of claim 22, 25, or 26 wherein the molecule capable of detecting Ang-2 polypeptide is selected from the group consisting of a Tie 1-Fc receptorbody polypeptide, a Tie 2-Fc receptorbody polypeptide, a Tie 1 receptor fragment polypeptide containing an Ang-2 binding domain, and a Tie 2 receptor fragment polypeptide containing an Ang-2 binding domain.

33. The kit of claim 22, 25, or 26 wherein the composition is administered to a mammal with a carrier suitable for parenteral administration.

34. The kit of claim 33 wherein the mammal is a human.

35. The kit of claim 23, 27, or 28 wherein the molecule capable of detecting Ang-2 nucleic acid is an mRNA.

36. The kit of claim 23, 27, or 28 wherein the molecule capable of detecting Ang-2 nucleic acid is a synthetic oligonucleotide.

37. The kit of claim 24, 28, or 30 wherein the molecule capable of detecting Ang-2 polypeptide is a synthetic polypeptide.

Figure 1A-1B

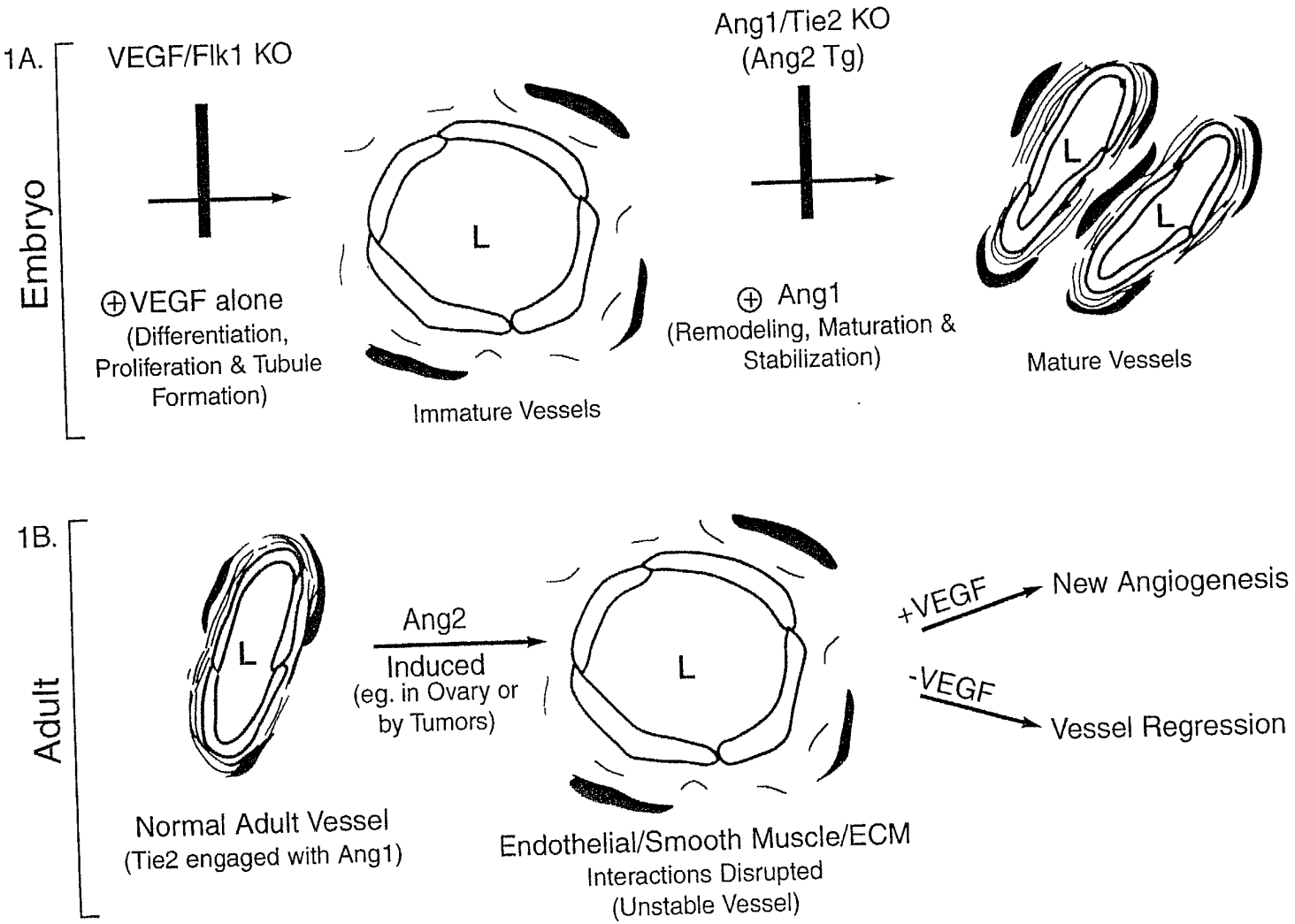


Figure 2A-2N

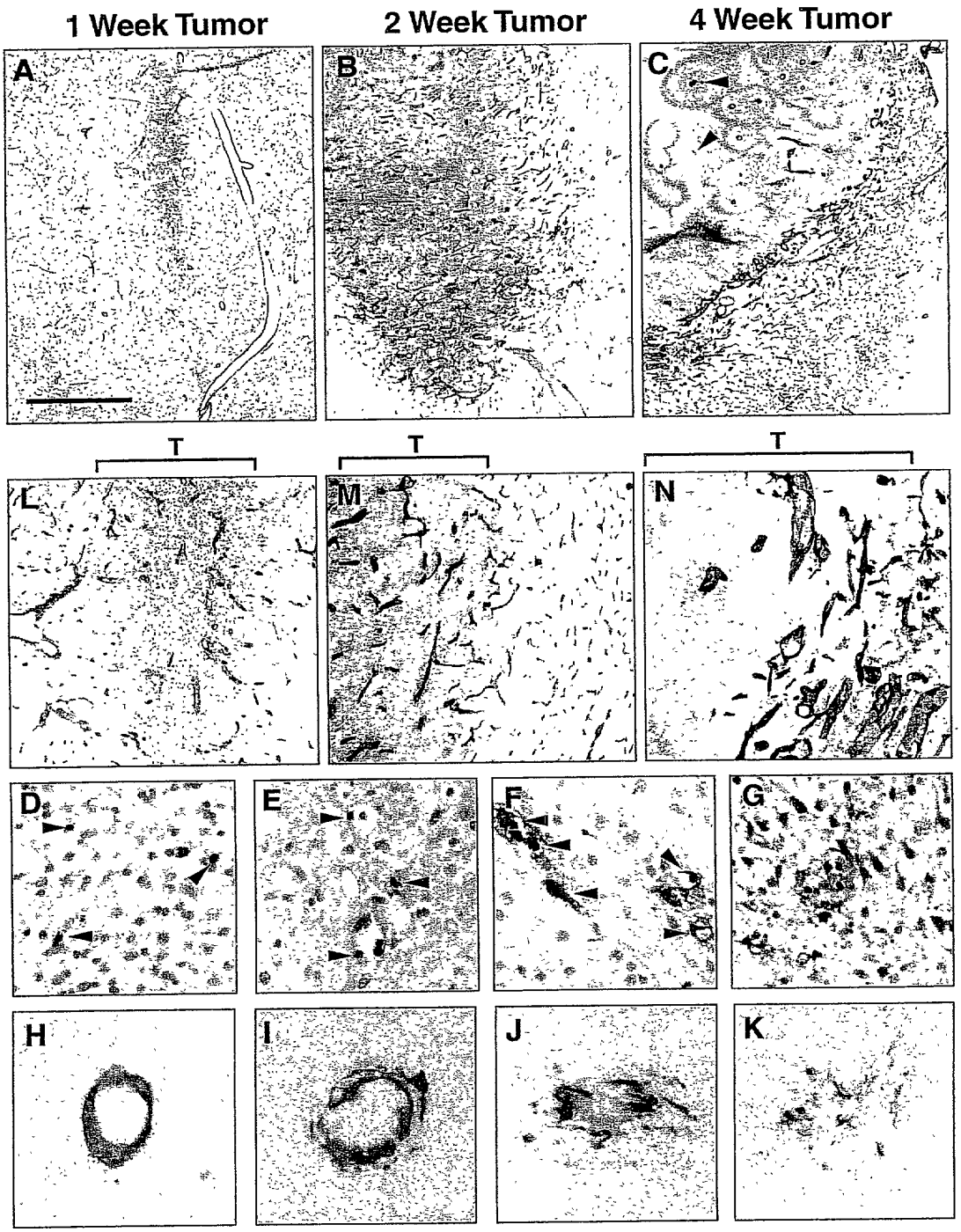


Figure 3A-3L

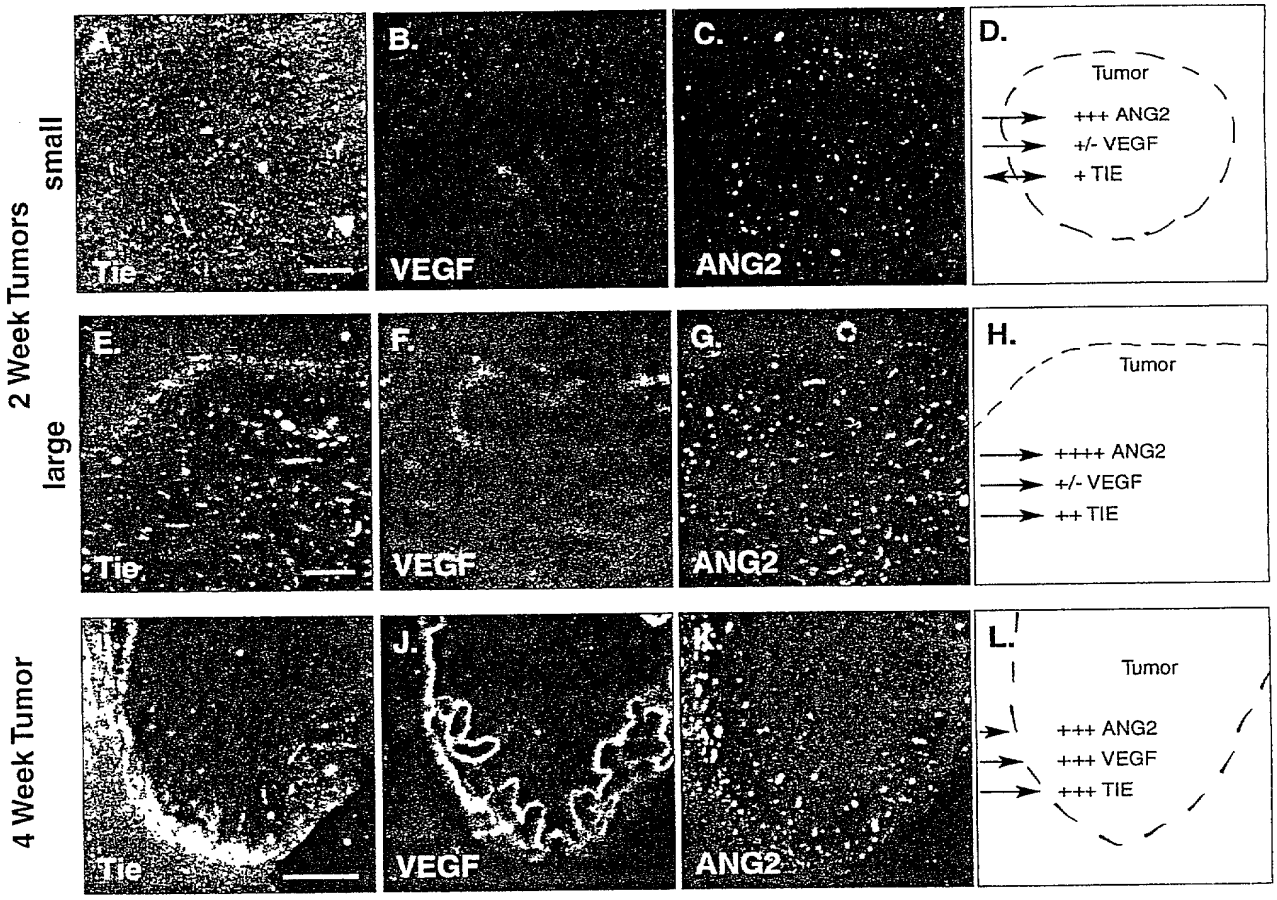


Figure 4A-4F

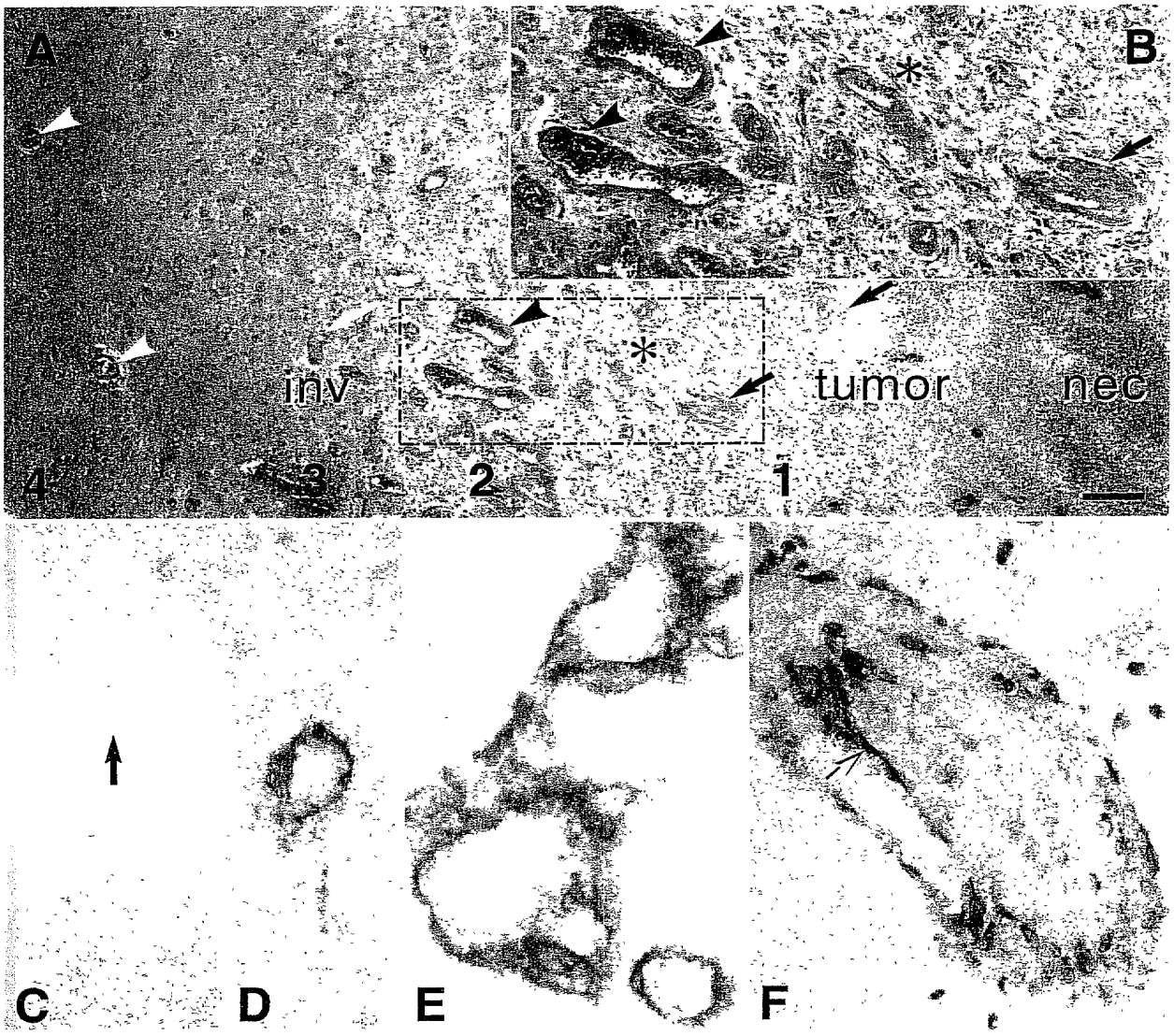
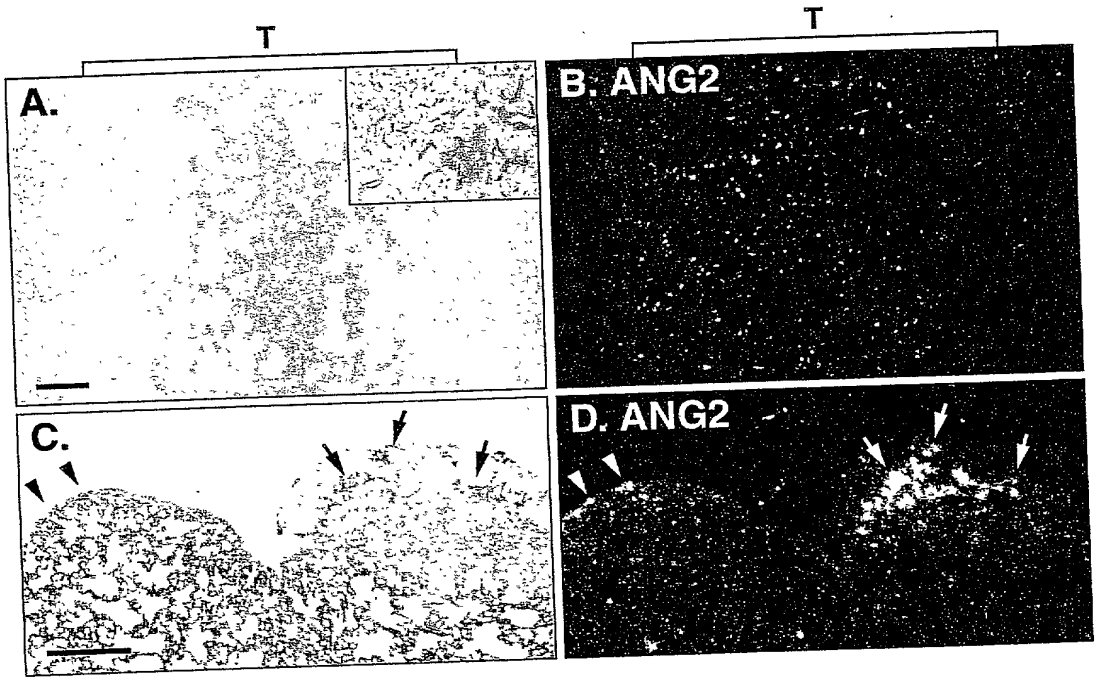


Figure 5A-5D



DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled **METHODS OF IMAGING AND TARGETING TUMOR VASCULATURE**, the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to in the oath or declaration.

I acknowledge the duty to disclose information of which I am aware that is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PCT/US00/15732 filed June 8, 2000

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States Application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) that occurred between the filing date of the prior application and the national or PCT international filing date of this application:

USSN 60/139,642 filed June 17, 1999

And I hereby appoint Joseph M. Sorrentino (Registration No. 32,598), Gail M. Kempler (Registration No. 32,143), Laura Fischer (Registration No. P-50,420), and Linda O. Palladino (Registration No. 45,636) each of them my attorneys and agent, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all

business in the Patent and Trademark Office connected therewith and to file any International Applications that are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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